Protocol:

**Evaluating the effect of hemodialysis modality on NETosis in hemodialysis patients**

Rationale: Patients suffering from chronic kidney disease (CKD) and diabetes mellitus (DM) are at an increased risk of developing cardiovascular complications and infections. Dysregulated NETosis may exacerbate pathogenic inflammatory pathways that are implicated in the complications of CKD, hemodialysis and diabetes.

Objectives: In light of the superior survival rates observed in patients treated with hemodiafiltration (HDF) compared to high flux hemodialysis (HFHD), alongside the documented dysregulation of NETosis in both hemodialysis and DM patients, this study aims to elucidate the effects of dialysis modality on NETosis activity in hemodialysis patients, stratified by diabetic status.

Methods: 40 hemodialysis patients will be recruited, comprising 20 diabetic and 20 non-diabetic patients. Blood samples will be collected from patients before and after HDF treatment, 1 week after transitioning to HFHD and after 3 weeks of HFHD. Neutrophils will be isolated and stimulated with 100 nM PMA for 1 hour or left without stimulation. Neutrophils will bestained for NETosis markers: Peptidylarginine deiminase 4 (PAD4), neutrophil elastase (NE), myeloperoxidase (MPO), Histone H3 and dsDNA. Data will be acquired using a flow cytometer. Serum levels of citrullinated histone H3 (citHIS), MPO and NE will bemeasured using ELISA.

**Expected Outcomes:**

We anticipate a significant increase in NETosis markers following high-flux hemodialysis (HFHD) treatment compared to hemodiafiltration (HDF) treatment. Additionally, NETosis markers are expected to exhibit a significant elevation in serum after 3 weeks of HFHD treatment. This notable increase in NETosis activation and markers after three weeks of HFHD treatment, compared to levels during HDF, underscores the role of HDF in attenuating dysregulated NETosis. These findings may translate to improved clinical outcomes in patients treated with HDF.

**General information**

- **Evaluating the effect of hemodialysis modality on NETosis in hemodialysis patients**
- The trial was registered and openly available at the Israel Ministry of Health website (https://my.health.gov.il/CliniTrials/Pages/Home.aspx) under reference number MOH_2022-09-22_012059, on 12/09/20222

- Sponsor: Galilee medical Center, Route 89 Nahariya, Israel, 22100

Funder: The Russell Barrie Galilee Diabetes- SPHERE Foundation

- Principal Investigator: Dr. Kruzel-Davila Etty, Director of the Nephrology Department, Galilee Medical Center

**Rationale & background**

Chronic kidney disease (CKD) is a significant global public health challenge, affecting an estimated 850 million people worldwide. Many of these patients progress to end-stage kidney disease (ESKD), requiring renal replacement therapies such as hemodialysis (HD) where such treatments are accessible. CKD patients face not only the burden of declining renal function but also a high prevalence of comorbidities and complications, particularly cardiovascular diseases (CVD) and infections.[1-3] CVD remains the leading cause of morbidity and mortality in this population, accounting for over 40% of deaths among dialysis patients. This elevated risk is driven by both traditional cardiovascular risk factors, such as hypertension and diabetes and CKD-specific factors, including accumulation of uremic toxins, fluid overload, and systemic inflammation. [1-3] In addition to CVD, CKD patients undergoing HD are highly susceptible to infections, which contribute significantly to morbidity and mortality. This vulnerability is partly due to secondary immunodeficiency related to kidney disease (SIDKD), frequent vascular access, and associated comorbidities. [1,4] Uremic toxins impair multiple immune functions, leading to reduced neutrophil activity,

impaired cytokine release, and dysfunction of natural killer, T and B cells. Chronic low-grade inflammation in uremic patients suppresses the immune response during infections, contributing to poor outcomes.[4] A key process in CKD-related inflammation is dysregulated NETosis, a form of neutrophil extracellular trap (NET) formation. NETosis is an evolutionarily conserved process aimed to entrap microorganisms. Neutrophils form these NETs by releasing decondensed chromatin (DNA coiled around histones) lined with the content of neutrophil intracytoplasmic granules.[5] In vitro, NETosis can be induced by phorbol-12-myristate-13-acetate (PMA) and requires activation of the Raf-MEK-ERK pathway along with NADPH oxidase-dependent production of reactive oxygen species (ROS). An increase in cytosolic calcium ions activates NADPH oxidase and functions as a cofactor for peptidylarginine deiminase 4 (PAD4), which catalyzes the citrullination of histone H3 (Cit-H3), leading to chromatin decondensation.[6] This process results in the extrusion of a mixture of DNA and bactericidal proteins, including myeloperoxidase (MPO) and neutrophil elastase (NE) and citrullinated histone H3 (citH3) all of which serve as markers of NETosis.[6,7] In addition to PMA, NETosis can also be triggered by antibodies, pro-inflammatory cytokines, chemokines, and sterile inflammatory stimuli such as high glucose levels, cholesterol, complement component C5a, and hypoxia.[6,7] Therefore, NETosis exhibits a dual-edged nature. While it was initially considered a protective host defense mechanism against pathogens, uncontrolled NET formation can lead to significant tissue damage, promoting necroinflammation. This switch from a beneficial response to a harmful one contributes to cardiovascular complications and a hypercoagulable state, particularly in patients undergoing HD. Dysregulated NETosis, potentially driven by bio-incompatibility during HD, is thought to contribute to the significant comorbid burden observed in this patient population.[7-12] Given the high cardiovascular and infection-related morbidity and mortality in ESKD patients undergoing HD, there is an urgent need to explore more biocompatible dialysis modalities. Hemodiafiltration (HDF) has emerged as a superior alternative to conventional high flux hemodialysis (HFHD). By combining diffusion and convection, HDF provides enhanced removal of larger uremic toxins implicated in inflammation and cardiovascular events.[13,14] Recent studies suggest that HDF may reduce cardiovascular morbidity, infection rates and improved survival, offering potential improvements in survival and quality of life for CKD patients.[15-17]

## Study goals and objectives

In light of the deleterious effects of enhanced NETosis during HD, we hypothesize that attenuated NETosis may contribute to the protective effects of HDF compared to HFHD. The combination of diffusion and convection in HDF is thought to mitigate oxidative stress and improve hemodynamic stability, leading to decreased NET formation during dialysis. This reduction in NETosis may improve immune function and reduce the risk of complications, ultimately enhancing survival outcomes in patients receiving HDF.

## Methods

### Study Design and Participants

Twenty hemodialysis patients who had been receiving HDF for at least three months, were included in this study, with 10 of the participants diagnosed with type 2 diabetes (T2DM) and 10 without. Participants were excluded if they had a history of autoimmune diseases, malignancies, chronic hepatitis B (HBV), hepatitis C (HCV), or HIV infection, or were taking medications known to directly affect the immune system. NETosis was assessed before and after HDF and again after one and three weeks of HFHD.

All participants provided written informed consent.

The study was approved by the Helsinki Committee at the Galilee Medical Center (Approval Number: 108-22-NHR).

### Blood Samples

Blood samples (EDTA tubes) and serum (clot activator tubes) were obtained from all participants before and after the 4-hour HDF treatment. After transitioning to HFHD, blood samples were collected in the same manner one week following HFHD treatment, with additional serum samples collected three weeks after HFHD treatment. Serum tubes were centrifuged at 4000 rpm for 10 minutes after clotting and were immediately stored at −80°C.

### Neutrophil Isolation

Neutrophils were purified directly from whole blood samples by immunomagnetic negative selection using the EasySep Direct Human Neutrophils Isolation Kit (STEMCELL

Technologies, UK), according to the manufacturer's instruction. Non-neutrophils cells were labelled with antibodies and removed using EasySep magnet. Isolated neutrophils were collected into a new tube and counted before culturing.

**Cell Culture and NETosis Assay**

Purified neutrophils (1 x $10^6$/ml) were seeded in a 24-well plate and incubated with RPMI 1640 medium (Sartorius, Ann Arbor, USA) containing 5% FBS HI (Gibco Fisher Scientific) at 37°C and 5% CO2. To assess NETosis, neutrophils were stimulated with 100 nM PMA (P8139; Sigma-Aldrich, St. Louis, MO) for 1 hour or left unstimulated.

**Flow Cytometer**

Following stimulation, neutrophils were collected and washed 3 times with staining buffer (PBS containing 1% FBS HI). Neutrophils underwent a standard cell staining protocol, without a permeabilization step, and incubated for 20 min at room temperature with tow separated mixes; (1) AF647 anti human PAD4 (sc-365369; Santa Cruz Biotechnology) and FITC anti human MPO (ab11729, Abcam, Cambridge, UK) (2) PE anti human NE (sc-55549; Santa Cruz Biotechnology) and AF647 anti human-Histone H3 (ab207543; Abcam). After 3 washes, 7-Amino-Actinomycin D (7AAD) Viability Dye for dsDNA staining (A07704; Beckman Coulter International, Nyon, Switzerland) was added to each mix for 20 min at room temperature. After incubation, suspended neutrophils were filtered through a 40 µm cell strainer (Falcon, BD Biosciences). Data acquisition was performed using the Navios Flow Cytometer (Beckman Coulter) and analyzed with Kaluza software version 2.1 (Beckman Coulter) (Supplementary Fig. 1).

**NETosis Markers in Serum Quantified by ELISA**

All serum samples were diluted 1:2 and citrullinated histone H3 (citH3) was quantified using the Citrullinated Histone H3 ELISA Kit (501620; Cayman Chemical, Ann Arbor, USA). Serum was diluted 1:1000 for MPO measurement using Myeloperoxidase ELISA Kit (501410; Cayman Chemical). NE was detected in serum (1:500 dilution) using Human Neutrophil Elastase ELISA Kit (ab204730; Abcam) according to the manufacturer's instructions.

**Safety considerations:**

Our dialysis patients are routinely treated with HDF. Conversion to high-flux hemodialysis for a duration of 3 weeks is not expected to cause any complications. Many dialysis units in Israel and other parts of the world regularly treat patients with high-flux hemodialysis, demonstrating its safety and routine use. Therefore, changing the dialysis modality for this short period is not anticipated to pose any harm or risk to the patients. However, it is important to note that no specific clinical benefits are expected from this temporary change.

**Follow-up**

This trial does not aim to explore clinical endpoints due to the short intervention period of switching to HFHD for only 3 weeks. No adverse events are anticipated as a result of this intervention. Upon completion of the study (3 weeks of HFHD), all patients will return to their regular HDF treatment.

**Data management and statistical analysis**

To ensure medical confidentiality, patients' details were stored in a file coded by serial numbers, without any identifying information. This coded file will be used exclusively for statistical processing. A separate file containing the coding and identifying details of the patients will be accessible only to the principal investigator and will not be used for data processing or collection.

The clinical and demographic data of diabetic and non-diabetic patients will be analyzed statistically. Quantitative variables were compared between the groups using the Mann-Whitney U test, while categorical variables were analyzed using Fisher's exact test.

For NETosis assays, statistical analyses and graph generation will be performed using Prism software version 2.1. All conditions will be compared using paired t-tests, or Wilcoxon tests for non-parametric data. Differences between subjects in the diabetic and non-diabetic groups will be assessed using unpaired t-tests. Statistical significance is defined as $p < 0.05$. Data are presented as mean ± SEM.

**Quality assurance**

The research is being conducted in compliance with the International Conference on Harmonization (ICH) Harmonized Tripartite Guideline for Good Clinical Practice (GCP) and in accordance with the Ministry of Health's procedures for conducting medical experiments. On-site monitoring visits will be carried out by the hospital's ethics committee to ensure adherence to these standards.

**Expected outcomes of the study**

We anticipate a significant increase in NETosis markers following high-flux hemodialysis (HFHD) treatment compared to hemodiafiltration (HDF) treatment. Additionally, NETosis markers are expected to exhibit a significant elevation in serum after 3 weeks of HFHD treatment. This notable increase in NETosis activation and markers after three weeks of HFHD treatment, compared to levels during HDF, underscores the role of HDF in attenuating dysregulated NETosis. These findings may translate to improved clinical outcomes in patients treated with HDF.

**Dissemination of results and publication policy**

After the publication of the article in professional journals, if the results have implications for public health, such as clarifying the protective mechanisms of HDF compared to HFHD through the reduction of NETosis, the hospital spokesperson will share the findings and their implications with the general public through local media and communication channels.

**Duration of the project**

The recruitment of patients is expected to take approximately six months. NETosis assays, which require the extraction of fresh neutrophils, will be performed on the same day the blood is drawn. Serum samples will be stored at -80°C, and the analysis of NETosis markers from the serum will be conducted after all blood samples have been collected. The total duration of the study, including data analysis, is anticipated to be around one to one and a half years.

**Problems anticipated**

If one of the participants develops an acute infectious disease, or is diagnosed with an autoimmune or malignant condition, their participation in the study will be discontinued, as these conditions may affect NETosis markers.

**Project management**

Dr. Etty Kruzel-Davila: Research idea and study design, data acquisition, data analysis/interpretation, supervision and mentorship.

Dr. Lital Remez-Gabay: Research idea and study design, data acquisition, statistical and formal analysis, data analysis/interpretation, supervision and mentorship.

Dr. Olga Vdovich: Data acquisition

Dr. Faten Y. Andrawes Barbara: Data acquisition

Dr. George Jiries: Data acquisition

**Ethics**

The study was approved by the Helsinki Committee at the Galilee Medical Center (Approval Number: 108-22-NHR).

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